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Effect of Estradiol Withdrawal on Luteal Hormone-Responsive Adenylyl Cyclase and Luteal Function in the Pseudopregnant Rabbit[1]

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ABSTRACT

We showed previously that continued administration of high levels of estradiol (E) to pseudopregnant (PSP) rabbits from Day 8 through Day 11 does not affect their serum progesterone levels, but leads to a 75% decrease in the ability of LH to stimulate the adenylyl cyclase enzyme in their corpora lutea (CL). To determine if the deleterious effect of estrogen is readily reversible, we now explored the effect of a 2-day withdrawal or a switch to low levels of E following the 3-day treatment with high E.

From 2200 h of Day 8 through 2200 h of Day 11, PSP rabbits were injected at 12 h intervals with 0.1 ml sesame oil alone (control), or with oil containing either 1.5 μg E (E-1.5) or 15 μg E (E-15). Several animals from each group were killed between 1000–1100 h the following morning (Day 12). The remaining animals were either switched from E-15 or E-1.5 to oil (E-withdrawal), were switched from E-15 to E-1.5, or were maintained on their original E regiments. The final injections were at 0800 h on Day 14 and the animals were killed 2–3 h later. Adenylyl cyclase activities and serum and luteal progesterone levels were determined.

We found that: 1) Withdrawal of E-15 did not reverse the E-15-induced inactivation of the LH-responsive adenylyl cyclase, nor did it alter the isoproterenol-responsive adenylyl cyclase. 2) Withdrawal of E-1.5 had no effect on the degree of hormonal responsiveness of the adenylyl cyclase when compared with control values. 3) E-15 withdrawal but not E-1.5 withdrawal, resulted in a significant decrease in serum and luteal progesterone content, but 4) did not result in a decrease in the weight of the CL within the 48-h time period tested. 5) Switching from E-15 to E-1.5 treatment did not cause a drop in serum progesterone concentrations, but 6) resulted in LH-stimulated cyclase activity, which although somewhat elevated over that found in animals treated with E-15 throughout, was still markedly decreased compared with controls or rabbits treated with E-1.5.

These results suggest that 1) a high dose of estradiol is required to establish a "dependency" on exogenous E in CL of PSP in rabbits; 2) low levels of exogenous E are sufficient to fulfill the requirements imposed by this E "dependent" state; and 3) the establishment of "dependency" on E is associated with the decrease in the LH-responsive adenylyl cyclase, an unphysiological situation. These data provide evidence that while a partial reversal of the suppression of cyclase activity by high levels of estradiol is possible, it proceeds only slowly taking several days to become effective.

INTRODUCTION

It has been well established that in the rabbit estradiol (E) is a primary luteotropic factor without which corpora lutea (CL) do not survive (Keyes and Nalbandov, 1967; Rennie, 1968). This is not to say, however, that under normal physiologic conditions of pseudopregnancy (PSP) or pregnancy, estradiol is the sole factor acting on CL and responsible for their maintenance. Specifically, it was recently shown by our laboratory (Hunzicker-Dunn and Birnbaumer, 1976a,b; Birnbaumer et al., 1976; Day and Birnbaumer, 1980a) that luteinizing hormone (LH) interacts directly with rabbit CL tissue to 1) stimulate adenylyl cyclase activity; 2) desensitize adenylyl cyclase activity; and 3) cause functional luteolysis in spite of a con-

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tinuous supply of E at a level that has been shown (Keyes and Nalbandov, 1967; Rennie, 1968) to be sufficient to maintain CL in the absence of follicular supply of E. This last finding strongly suggested that proper functioning of the CL adenylyl cyclase is also required for luteal function.

In a previous study in which PSP rabbits were treated with low (1.5 μg twice daily) or high (15 μg twice daily) doses of E, it was discovered that high E alone, while not affecting serum progesterone levels significantly, led to alterations in the LH-stimulable adenylyl cyclase of CL. The alteration noted consisted in a significant (75%) loss of LH-stimulable activity within 2 days of treatment without loss of either basal or isoproterenol-stimulated activity. No such deleterious effect on the ability of LH to stimulate adenylyl cyclase activity was noted in animals treated with low E. It became of interest to evaluate whether the effect of high E was permanent or readily reversible upon withdrawal of the steroid. The present study reports the results of such experiments. We found that withdrawal of exogenously administered high E or replacement with low physiological doses of E does not result in rapid reversal of the effects on adenylyl cyclase, and that in accordance with findings by Holt et al. (1975), withdrawal is accompanied by a loss in progesterone secretion from CL. Further, when high E was switched to low E treatment, serum progesterone did not decline, indicating that the high E treatment had made CL dependent on exogenous E.

MATERIALS AND METHODS

Materials

Estradiol (Sigma Chemical Co.) was dissolved in sesame seed oil. LH (NIH-LH-S19, obtained from NIAMDD) was kept as a stock solution (1 mg/ml) in 0.15 M NaCl. (−)-Isoproterenol was a gift from Dr. F. C. Nachod (Sterling Winthrop Research Institute) and kept as a 10^{-1} M stock solution in 10^{-3} M HCl. For adenylyl cyclase assays, LH and isoproterenol stock solutions were diluted with 0.1% bovine serum albumin to 50 μg/ml and 5×10^{-4} M, respectively; 10 μl of each solution were used in the assays. Creatine phosphate and creatine kinase were from CalBiochem; myokinase, ATP(Tris-salt), cAMP, EDTA and Tris were from Sigma Chemical Co. [α-^{32}P]-ATP (20–50 Ci/mmol), synthesized according to Birnbaumer et al. (1979), was supplied by the Core Laboratory on Cyclic Nucleotide Research, Center for Population Research and Studies on Reproductive Biology, Baylor College of Medicine, TX. [^{3}H]-cAMP (10–15 Ci/mmole) was from Amersham-Searle. All other chemicals and reagents were of the highest commercially available purity and were used without further purification.

Animals

New Zealand white rabbits (3.5–4.5 kg), which had littered at least once, were housed in individual cages in air-conditioned quarters and allowed free access to water and a commercially pelleted food for at least 15 days before initiation of experiments. Such females, if not pregnant, were considered to be in estrus. Pseudopregnancy was induced by injecting into the marginal ear vein 100 IU hCG (donated by Dr. J. Jewell, Ayerst) dissolved in 0.5 ml 0.9% saline. The day following hCG injection was designated Day 1 of PSP.

Treatments and Preparation of Sera and CL Homogenates

Starting at 2200 h on Day 8 of PSP, rabbits received at 12 h intervals 0.1 ml sesame seed oil, s.c., without (control) or with 1.5 μg E (E-1.5 treatment) or 15 μg E (E-15 treatment). In E withdrawal experiments, the last injection was at 2200 h on Day 11 of PSP; the animals were killed on the morning of Day 12 (control) or Day 14 (withdrawal). Other rabbits continued to receive E treatment at the original doses or were switched from E-15 to E-1.5 beginning at 0800 h of Day 12. These rabbits received the final E injection at 0800 h on Day 14, 15, or 16 of PSP and were killed 3 h after the final injection.

The rabbits were bled from the marginal ear vein on Day 12 and on the day of sacrifice. The animals were killed by cervical dislocation between 1000–1200 h. The ovaries were removed immediately and cooled to 0–4°C in iced Krebs-Ringer bicarbonate buffer prepared with one-half the recommended amount of $CaCl_2$ (Cohen, 1957). The CL were dissected free of interstitial tissue using Graefe forceps (Roboz Surgical Co.) and kept in iced Krebs-Ringer bicarbonate buffer until further processing (30 min–1 h). Prior to homogenization, CL were blotted and weighed. Homogenization was performed in 10 volumes of ice cold 27% wt/wt sucrose in 1 mM EDTA and 10 mM Tris HCl, pH 7.5, as previously described by Hunzicker-Dunn and Birnbaumer (1976a), followed by a 2-fold dilution with the same homogenizing medium. Homogenates were analyzed for adenylyl cyclase activity within 30 min and for progesterone content after storage at −20°C for up to 6 months. Pooled CL from a single rabbit yielded enough homogenate for triplicate determinations of basal, LH-stimulated and isoproterenol-stimulated adenylyl cyclase activities, respectively, as well as for duplicate determinations of protein and 2 triplicate assays of progesterone.

Adenylyl Cyclase Assays

Adenylyl cyclase activity in 20 μl aliquots of homogenate was determined as described earlier (Hunzicker-Dunn and Birnbaumer, 1976a) at 32.5°C in 50 μl medium containing 3.0 mM [α-^{32}P]-ATP ($5–15 \times 10^{6}$ cpm), 5.0 mM $MgCl_2$, 1 mM EDTA, 1 mM [^{3}H]-cAMP (∼10,000 cpm), 20 mM creatine

phosphate, 0.2 mg/ml creatine kinase, 0.02 mg/ml myokinase and 25 mM Tris HCl. When present, LH was 10 μg/ml and isoproterenol was 10^{-4} M. The final pH of the incubation (10 min) was 7.0. The [^{32}P]-cAMP formed was isolated by the method of Salomon et al. (1974) as modified by Bockaert et al. (1976). Under the conditions employed, adenylyl cyclase activities were linear with respect to time of incubation for up to 20 min and with respect to homogenate concentration for up to the equivalent of 40 μl of 1:20 homogenates (i.e., 20 μl of homogenates prepared by homogenizing 1 part of CL in 10 parts of homogenization medium and omitting the subsequent 1:2 dilution step). Results are expressed as pmoles of cAMP formed/min/mg protein.

Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin (Fraction V, Armour) as standard.

Progesterone Assays

Solutions, extractions of sera and homogenates, setup of the radioimmunoassay (based on antiserum GDN #337 supplied by Dr. Gordon D. Niswender), separation of free from bound progesterone, estimation of recoveries and evaluation of results were described in detail elsewhere (Day and Birnbaumer, 1980a,b). The characteristics of this antibody have been published by Gibori et al. (1977) and confirmed by us.

Statistical Analyses

Significance of differences between groups was calculated by Student's t test.

RESULTS

The effect on hormone-sensitive CL adenylyl cyclase of a 3 day treatment of PSP rabbits with oil, 1.5 μg E twice daily (E-1.5 treatment) or 15 μg E twice daily (E-15 treatment), followed by a 2-day withdrawal period, is shown in Fig. 1. Low E (E-1.5) treatment had no significant effect on any of the parameters tested ($P>0.1$) when compared with results obtained in control (oil-treated) rabbits. High E (E-15) treatment, however, resulted in a selective decline in LH-stimulable adenylyl cyclase activity from 74.0 ± 10.2 to 18.7 ± 4.9 pmols cAMP formed/min/mg homogenate protein (± SEM, $P<0.001$). Withdrawal of E for 48 h did not result in return to control values (LH-stimulable activity in homogenates of 14-day-old CL [pmols/min/mg ± SEM]: control, 49.1 ± 2.32; E-15, 23.3 ± 1.44; $P<0.005$). Interruption of E-1.5 treatment, however, resulted in a slight (36%, $P<0.05$) increase in LH-stimulable adenylyl cyclase in homogenates of 14-day-old CL when compared with control values. In these experiments, basal and isoproterenol-stimulated activities were not affected by either one of the E treatments or the following withdrawals. On Day 12 of PSP, there were no significant differences among the treatment groups in CL weight, in CL progesterone content, or in serum progesterone (Table 1; Figs. 2, 3).

Although high E treatment did not alter serum or luteal progesterone levels, withdrawal of exogenous high E resulted within 2 days in a decrease in serum progesterone levels to 18% of control levels ($P<0.05$), or to 15% of the levels found in the low E-withdrawal group ($P<0.005$, Fig. 2). In neither the oil-treated group nor the low E-withdrawal group did the serum progesterone level fall between Day 12 and Day 14 of PSP. Tissue levels of progesterone fell only in the high E-withdrawal group ($P<0.01$, Fig. 3).

The weight of the CL on Day 14 from the high E-withdrawal group did not differ from the weight of the CL from the control group (Table 1). There was a small, but not significant, decline in CL weight between Day 12 and Day 14 in the high E-withdrawal group ($P>0.1$).

Table 2 contains data showing stimulation of the cyclase relative to basal levels by LH, isoproterenol and NaF in two different experiments. In experiment 1, estradiol treatment was terminated on Day 12 while in experiment 2 it was altered or continued until Day 14. As shown in Table 2, on Day 14, whereas the relative stimulation of the cyclase by LH was slightly greater in the E-15 group that had been switched to E-1.5 treatment compared with the group receiving only E-15 treatment ($P<0.05$), it was still significantly depressed when compared with controls or with the group receiving only E-1.5 treatment ($P<0.01$).

The mean serum progesterone concentrations were not different in any of the groups of rabbits receiving E through Day 14 and were similar to those in rabbits on Day 12. In additional experiments we prolonged the treatments with high or low E, after initial 3-day E treatments, for 1 or 2 more days (i.e., the animals were killed on Days 15 or 16 of PSP). However, serum progesterone concentrations fell to basal by Day 15 in most of the rabbits switched from E-15 to E-1.5 treatment. Similarly, serum progesterone levels fell to basal values by Day 16 in rabbits receiving E-1.5 treatment throughout. An interesting and somewhat unexpected finding was that the mean serum progesterone concentration of rabbits receiving E-15 treat-

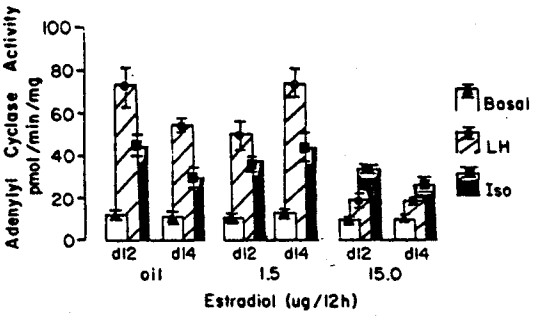

FIG. 1. The effect of E treatment and its withdrawal on adenylyl cyclase activities in 12- or 14-day-old CL from pseudopregnant rabbits that received E until 2200 h on Day 11. After sacrifice on Day 12 or 14, luteal homogenates were made and assayed as described under Materials and Methods. When present in the assay, LH (NIH-LH-S19) was 10 μg/ml and isoproterenol (Iso) was 10^{-4} M. Results are expressed as means ± SEM with 3 animals/group.

ment throughout was still somewhat elevated on Day 16 (7 ng/ml).

Based on serum progesterone concentrations, luteal regression was evidently underway by Day 15 or 16. Therefore, statistical analysis of the data from animals killed on Day 15 or 16 was not feasible. However, the following trends could be noted in rabbits with serum progesterone concentrations above 4 ng/ml. In animals treated with E-15 throughout, the LH-responsive cyclase activities on Days 12, 14, 15 and 16 were similar and were consistently less than the respective cyclase activities in rabbits treated with E-1.5 throughout. The LH-responsive adenylyl cyclase activities in the rabbits switched from high to low E were intermediate between the E-15 and E-1.5 treated rabbits but never returned to the level found with E-1.5 treatment throughout.

DISCUSSION

The results presented above indicate that treatment of PSP rabbits with low doses of E capable of maintaining either in situ CL (Keyes and Nalbandov, 1967) or ectopic CL (Rennie, 1968) in the absence of follicular E supply, has no deleterious effect on CL adenylyl cyclase and its hormonal responses or on CL function as assessed by determination of luteal and serum progesterone levels. Interruption of low E treatment had no noticeable consequence. However, treatment with a 10-fold higher dose of E resulted in partial loss of the ability of LH to stimulate adenylyl cyclase activity and, upon interruption of E administration, in loss of progesterone production without a return of ability of LH to stimulate activity of CL adenylyl cyclase. Even when the E-15 was switched for 2 days to the E-1.5 treatment, a procedure that maintained high progesterone levels, the ability of LH to stimulate adenylyl cyclase activity was not restored to control values. This indicated that alterations in the LH-responsiveness of the cyclase induced by the high E treatment were not readily reversible despite the slight increase in LH-responsiveness observed after the switch from high to low E. The significance of this slightly increased responsiveness of the cyclase after the switch from high to low E is not clear. It could reflect an effect of E-1.5 treatment at that particular time of the CL lifespan or it could mark the initiation of reversal of the effects of high E. Although the data from animals switched from high to low E would suggest that the high E induced permanent changes in the cyclase, a more definitive answer will have to come from a different experimental protocol since our inability to restore the LH-stimulable adenylyl cyclase to normal levels by Days 15 or 16 may

TABLE 1. Effect of estradiol withdrawal on weight of corpora lutea of PSP rabbits.

Treatment[a]	Weight of corpora lutea[b] (mg/CL)	
	Before withdrawal (12-day-old CL)	After a 2-day withdrawal (14-day-old CL)
Control (oil)	21.0 ± 0.5 (6)	18.5 ± 2.3 (3)
E-1.5	19.1 ± 1.1 (6)	21.2 ± 3.0 (3)
E-15	21.9 ± 0.9 (6)	17.7 + 0.6 (3)

[a]For details of treatments see Materials and Methods and Fig. 1.

[b]Means ± SEM. The number between parentheses indicates n rabbits from which 8–14 CL were removed and weighed.

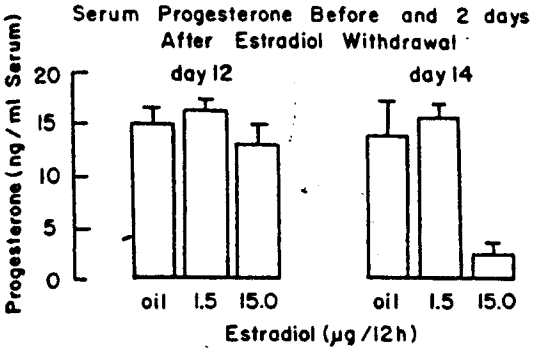

FIG. 2. Serum progesterone levels on Day 12 in E-treated pseudopregnant rabbits and on Day 14 in E-treated and then withdrawn rabbits. Sera were collected from the marginal ear vein and assayed for progesterone by radioimmunoassay. Further details of the treatments and the assay conditions are described under Materials and Methods. Results are expressed as the mean ± SEM. Sera were from 6 rabbits in each of the 12-day PSP groups and from 3 rabbits in each of the 14-day PSP groups.

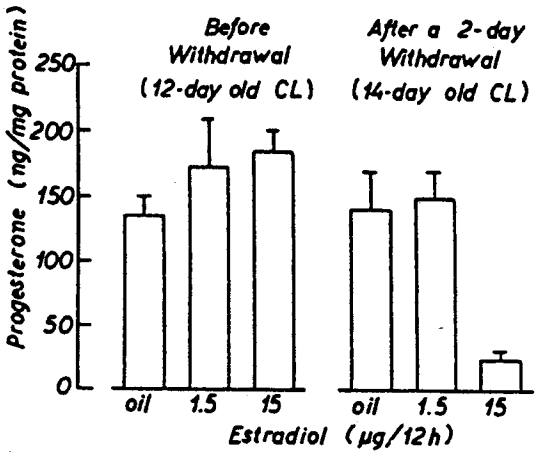

FIG. 3. Progesterone content of Day 12 CL from estrogen treated rabbits and of Day 14 CL from estrogen treated and then withdrawn rabbits. Further details of the treatment schedule and the assay of CL homogenates for progesterone are described under Materials and Methods. Results are expressed as the mean ± SEM with 3 animals/group.

be either a result of luteal regression or a result of estrogen treatment.

The means by which E leads to such a permanent decrease in the LH-responsive adenylyl cyclase system remains unknown, although two possibilities appear feasible. Estrogen treatment may result in an endogenous LH surge thus inactivating the LH-responsive adenylyl cyclase, or estrogen may act directly to alter the LH-receptors or their ability to couple to the adenylyl cyclase. Support for the former possibility comes from findings of Dufy-Barbe et al. (1978) demonstrating that in the rabbit E can elicit an LH peak, and from our findings showing that LH or hCG can desensitize the LH-responsive adenylyl cyclase in rabbit CL (Hunzicker-Dunn and Birnbaumer, 1976b; Day and Birnbaumer, 1980a). However, ovulatory doses of LH or hCG are required (Hunzicker-Dunn and Birnbaumer, 1976b). In the present study, no new ovulatory points were seen, suggesting that if an LH surge did occur, it was subovulatory or that the follicles were less sensitive to the surge.

The effect of estrogen on the CL could also be direct. Three-day-old CL that have been exposed to high E for 3 days remain responsive to LH (Day and Birnbaumer, 1980a). These CL have not yet acquired a significant number of estrogen receptors which appear by Day 4 (Mills and Osteen, 1977). This appearance of receptors precedes the development of estrogen dependence in rabbit CL (Miller and Keyes, 1977) which is clearly established by Days 5–6. It is possible that upon treatment with high E, CL become dependent on continuous E supply secondarily and consequent to the E-induced decrease in the ability of LH to stimulate activity of adenylyl cyclase, the decrease being a receptor-mediated event. Steroid hormones are known to alter gene expression and consequently protein synthesis (O'Malley and Means, 1974). Treatment with high E may, therefore, alter LH responsiveness either by diminishing the number of LH receptors or by modifying receptor-cyclase coupling. The cyclase system itself appears to be untouched since in our experiments both basal and isoproterenol-responsiveness do not change with high E treatment. Although estrogen administration in vivo has been reported to stimulate hCG binding to luteinized rat ovarian slices (Lee and Ryan, 1974), this effect might be mediated via prolactin (Holt et al., 1976). Thus, we are investigating both the possibility of decreased receptor and of decreased coupling of the hormone receptor complex to the adenylyl cyclase system. It is of interest to note that during the first 3 days of PSP in the rabbit, the LH-responsive adenylyl cyclase activity reaches a peak and then declines (Hunzicker-Dunn and Birnbaumer, 1976a). Whether this elevated activity reflects a lack of control by estrogen remains to be seen.

TABLE 2. Comparison of effect of continuous estradiol treatment with effect of estradiol withdrawal. Mean ± SEM.

	Treatment			Stimulation relative to basal		
	Days 8–11	Days 12–14	n	LH	Isoproterenol	NaF
Experiment 1	E-15	Oil	3	1.86 ± 0.29[a]	2.58 ± 0.46	...
	E-1.5	Oil	3	5.88 ± 0.45[b]	3.50 ± 0.49	...
	Oil	Oil	3	4.75 ± 0.84[b]	3.06 ± 0.68	...
Experiment 2	E-15	E-15	4	1.89 ± 0.24[a]	3.89 ± 0.21[a]	6.15 ± 0.58
	E-15	E-1.5	4	2.75 ± 0.09[b]	2.99 ± 0.14[b]	6.04 ± 0.87
	E-1.5	E-1.5	4	4.51 ± 0.59[c]	3.66 ± 0.28	5.07 ± 0.63

[a]Groups with different superscripts within the same experiment are significantly different at at least the $P<0.05$ level.

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